Education of Chondrogenic Mesenchymal Cells to Acquire Articular-like Permanent Cartilage-Forming Activity

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INTRODUCTION: Chondrogenesis from human pluripotent stem cell (hPSC)-derived mesodermal cells serves as a valuable human model for cartilage development. These mesodermal cells can be expanded through the application of exogenous growth factors, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), along with inhibitors, SB431542 to counteract transforming growth factor-beta (TGF-β), and CHIR99021 to activate canonical WNT signaling [1-3]. However, changes in the medium composition result in two distinct types of chondrogenic mesenchymal cells: those expressing SOX9 ($SOX9^+$), and those expressing GDF5 ($GDF5^+$) and tendon/ligament markers such as *SCX*, *MKX*, and *TNMD*. Interestingly, these two types of cells preferentially give rise to different types of chondrocytes: i.e., endochondral ossification-prone and -resistant chondrocytes from the former and the latter , respectively [3]. Notably, cartilage pellets derived from $SOX9^+$ cells readily mineralize, while those from $GDF5^+$ cells remain unmineralized for up to 8 weeks *in vivo* [3]. Comparative transcriptome analyses suggest distinct endogenous signaling mechanisms at play during development of each cell type: bone morphogenetic protein (BMP), retinoic acid (RA), Neuregulin (NRG), and FGF signaling in $SOX9^+$ cells, and Activin/TGF-β, Interleukin (IL)-6 family cytokine, such as IL-11 and Leukemia Inhibitory Factor, Neurotrophin (NT), and PDGF signaling for $GDF5^+$ cells (Fig. 1). We propose that these unique endogenous signaling mechanisms influence the hPSC-derived mesodermal cells to acquire different chondrogenic activities in the $SOX9^+$ and $GDF5^+$ progeny.

METHODS: To test this hypothesis, we treated the mesodermal cells during expansion culture as well as differentiation cultures toward $SOX9^+$ cells and $GDF5^+$ cells with activators and inhibitors of the predicted signaling mechanisms to assess their effects on chondrogenic properties of resulting $SOX9^+$ cells and $GDF5^+$ cells. First, hPSC lines were differentiated toward paraxial mesodermal progeny in a chemically-defined medium (CDM) as previously described [1-3]. Then, we isolated the mesodermal fraction using cell sorting [3, 4]. These mesodermal cells were maintained in CDM containing FGF2, PDGF, SB431542 and CHIR99021 and $SOX9^+$ mesenchymal cells were conventionally generated by removing PDGF [3]. To produce $GDF5^+$ mesenchymal cells, we transferred the mesodermal medium to CDM supplemented with PDGF and Noggin (a BMP inhibitor) [3]. Chondrogenesis was performed using PDGF, TGF-β3 and BMP4 as described previously [1-3], and *in vitro* generation of endochondral ossification-prone hypertrophic chondrocytes (i.e., [$COL2A1^+$] $COL10A1^+PRG4^{lo}$) and permanent/primitive articular-like chondrocytes (i.e., [$COL2A1^+$]$COL10A1^{lo}PRG4^+$) was demonstrated by RT-PCR analyses.

RESULTS: Generally, cartilage pellets generated from $GDF5^+$ cells express *COL10A1* at lower (1-10%) levels, and *PRG4* at higher levels than those from $SOX9^+$ cells *in vitro* [3]. Previous research showed that IL-6 family cytokine and Activin/TGF-β signaling enhanced *GDF5* and *SCX* expression, respectively, during $GDF5^+$ cell genesis, but they did not significantly affect $COL10A1^{lo}$ permanent-like chondrocyte formation from the developed $GDF5^+$ cells [5], suggesting that the mesenchymal cell markers are not proper predictors for their biological properties. Similarly, manipulation of endogenous signaling mechanisms predicted to be functioning during $GDF5^+$ cell genesis did not significantly affect the $COL10A1^{lo}PRG4^+$ cartilage forming activity in the resulting $GDF5^+$ cells (data not shown). However, treatment of mesoderm while expanded, specifically with inhibitors BMP signaling and RA signaling, led to cells during the conventional $GDF5^+$ cell genesis culture which show an improved chondrogenesis capacity to generate $COL10A1^{lo/-}PRG4^{+/hi}$ cartilage (lower *COL10A1* and higher *PRG4* levels, green squares in Fig. 2A). In addition, weak enhancement of BMP and NRG signaling during the genesis culture for $SOX9^+$ cells resulted in cells showing improved capacity to form $COL10A1^+PRG4^{lo}$ cartilage, but the effects were weak and not reproducible (data not shown). However, the same treatment during the mesoderm expansion culture resulted in $SOX9^+$-like cells that showed clear improvement of their chondrogenic activity to develop $COL10A1^{+/hi}PRG4^{lo/-}$ cartilage (higher *COL10A1* and lower *PRG4* levels, red squares in Fig. 2B).

DISCUSSION: While our previous RNA-seq analyses of the $SOX9^+$ and $GDF5^+$ cells [3] predicted many potential endogenous signaling mechanisms (Fig. 1), the most significant is thus far BMP and NRG signaling that enhanced the $COL10A1^+PRG4^{lo/-}$ chondrocyte forming activity in mesodermal cells and inhibition of BMP and RA signaling in mesodermal cells are sufficient to improve the capacity of $GDF5^+$ cells differentiated from such cells to form $COL10A1^{lo}PRG4^+$ primitive articular-like chondrocytes. These results suggest that educating early developmental stage cells (mesodermal cells) has sustained effects on their progeny's biological property (Fig. 3). We plan to perform single cell RNA-seq analyses to determine which subpopulation of treated and untreated mesodermal cells will lead to $GDF5^+$ cells and is responsible for permanent cartilage-forming activity.

SIGNIFICANCE/CLINICAL RELEVANCE: Our results support our hypothesis that the fate of chondrocytes originating from chondrogenic mesenchymal cells can be predetermined during the mesenchymal cell stage, which seems to depend on how the mesenchymal cells are educated during their maintenance/expansion cultures. Further biological studies will have profound implications for enhancing the potential of hPSC-derived mesenchymal chondroprogenitors in cartilage regenerative therapy. Moreover, these insights might extend to improving cell-based cartilage therapy involving adult chondrogenic cells, such as bone marrow mesenchymal stromal cells, by applying similar mechanisms to improve therapeutic outcomes.

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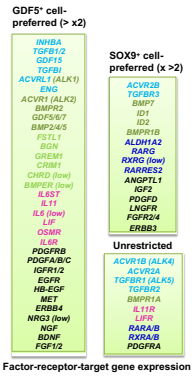

Fig. 1. *Potential endogenous signaling mechanisms for $SOX9^+$ cells and $GDF5^+$ cells.*

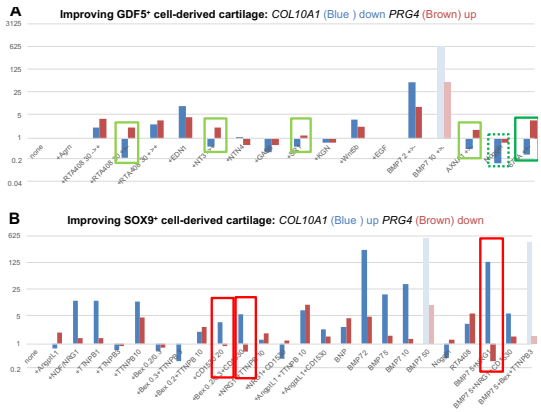

Fig. 2. *Determination of endogenous signaling mechanisms critical for the permanent cartilage forming activity of $GDF5^+$ cells.* +>-: treated mesoderm culture for 7 days, then untreated $GDF5^+$ cell genesis culture. ->+ vice versa, +>+ both stages treated

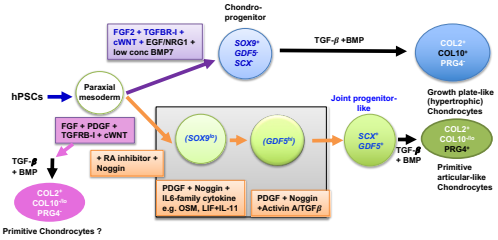

Fig. 3. *Working model for development of $SOX9^+$ and $GDF5^+$ cells.*